REDUCING HAIR GROWTH, HAIR FOLLICLE AND HAIR SHAFT SIZE AND HAIR PIGMENTATION

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This application claims the benefit of U.S. Provisional application 60/145,774, filed 07/27/1999.

AKS 6/5/01

Field of the Invention

This invention is related to methods and compositions effective for reducing hair growth. More specifically, the present invention is directed to methods for changing the rate of hair growth, reducing the size of the hair follicle and the hair shaft, and reducing hair shaft pigmentation, by topical application of either botanical extracts containing serine protease inhibitory activity and in particular soybean extracts such as soymilk, or mixtures and formulations of the above, combined with other active ingredients such as isoflavones.

Background of the Invention

One main function of mammalian hair is to provide environmental protection. However, that function has largely been lost in humans, in whom hair is kept or removed essentially for social and cosmetic purposes.

Many procedures are used to remove unwanted hair including shaving, electrolysis, plucking, laser and light therapies and injection of therapeutic antiandrogens. These conventional methods are not without their shortcomings. Shaving, for instance, may result in nicks and cuts in the skin's surface, may leave a perception of an increase in the rate of hair

regrowth, and may also leave undesirable stubble. While electrolysis may keep an area free of unwanted hair for a prolonged period of time, the process is often expensive and painful and may further result in scarring. Not only may plucking cause pain and discomfort, but it often result in a poor removal of short hair. Several unwanted side effects, such as effects on muscularity, often accompany the use of antiandrogens. For these reasons, better methods for reducing hair growth are needed.

Pseudofolliculitis barbae is an inflammatory hair disorder, most commonly found on the beard area. Inflammatory follicular papules result when hair tips penetrate into the skin rather than passing through the follicular orifice. This process is extremely common in black men because their hairs are frequently curly, exiting the skin at an acute angle. Close shaves, particularly with a razor blade, predispose them to pseudofolliculitis barbae. The most effective treatment available is to allow the hairs to grow well beyond the skin surface. Such a treatment is often not desired.

Hirsutism is a relatively frequent condition affecting about 4% of women. Facial hirsutism often interferes with personal and work activities, and temporary hair removal is a major component in the management of hirsute patients. Shaving is the most

frequently used temporary method for facial hair, as plucking, waxing and depilatories are more difficult to tolerate and care must be taken to avoid folliculitis, pigmentation, and scarring. Cosmetic cover-ups are usually used to hide cuts and stubble and electrolysis and thermolysis may be used for permanent hair removal when affordable.

An alternative or complementary desired approach to hair removal, would be a method to reduce hair growth, reduce hair follicle and hair shaft size and reduce hair shaft pigmentation. Such an approach could reduce the visibility of existing hair, making it softer and lighter. When combined with other methods of hair removal such a method could enhance and prolong the removal effect, and reduce the need and frequency of hair removal. Long term use of such an approach could lead to attenuated, soft, pigmentation-reduced hair growth, that is less visible and does not require the use of other removal methods.

Reduced hair growth is desired in the axilla area (fossa axillaris), where deodorants and anti-perspirants are used to control odor trapped within the axillary hairs. It would be desired to have products for under-arm use, which combine deodorant or anti-perspirant activities with reduced hair growth activity.

African type hair is unique in its morphology -- a kinky hair shaft with variations in diameter. This complex shaft structure creates the need for specialized grooming products and procedures to ensure that the African type hair maintains cosmetic desired properties. It is desired to have products that reduce this complexity and make the African type hair more manageable, improving its appearance.

It would be desirable to provide a method for chemically or naturally affecting hair growth, hair follicle and hair shaft size and hair shaft pigmentation, which does not cause unwanted side effects to the user.

Summary of the Invention

In accordance with the present invention, we have found compositions and methods for affecting changes in mammalian hair growth, hair follicle and hair shaft size and hair pigmentation by topically applying to the skin of a mammal an effective amount of a topically active composition comprising protease inhibitors, botanical extracts, and in particular legume extracts including, but not limited to, soymilk, for a period of time sufficient to affect hair growth, hair follicle and hair shaft size and hair shaft pigmentation. Such topically active compositions may be further combined with other active ingredients

including, but not limited to, synthetic or naturally occurring isoflavones, to enhance the desired effects on hair growth and pigmentation.

The compositions and methods of this invention provide a unique, convenient means for delaying hair growth, reducing hair follicle and hair shaft size and hair shaft pigmentation, by using serine protease inhibitors, botanical extracts of the legume family, and in particular, but not limited to, soymilk, containing serine protease inhibitory activity, and their combinations with isoflavones.

Brief Description of the Drawings

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

The invention will be more fully understood and further advantages will become apparent when reference is made to the following detailed description of the invention and the accompanying drawings in which:

Figure 1: A photograph of control and soymilk treated C57Bl/6 mouse hair (high magnification).

Figure 2: Histological sections of control and soymilk-treated C57Bl/6 mouse hair follicles at day four of the hair cycle.

Figure 3: Histological sections of control and soymilk-treated C57Bl/6 mouse hair follicles at day seven of the hair cycle, high and low magnifications.

Figure 4: Histological sections of control and soymilk-treated C57Bl/6 mouse hair follicles at day 18 of the hair cycle.

Figure 5: Histological sections of control and soymilk-treated C57Bl/6 mouse hair follicles at day 21 of the hair cycle.

Figure 6: Histological sections of control, soymilk, and soymilk-derived proteins-treated C3H mouse hair follicles (High magnification) at day seven of the hair cycle.

Figure 7: Histological sections of control, soymilk, and soymilk-derived proteins-treated C3H mouse hair follicles (lower magnifications) at day seven of the hair cycle.

Figure 8: Histological sections of control and soymilk-treated C3H mouse hair follicles at day 21 of the hair cycle.

Figure 9: A photograph of control and soymilk-derived proteins treated C3H mouse hair (high magnification).

Figure 10: Histological sections of control and soymilk-derived proteins-treated C57Bl/6 mouse hair follicles (High magnification) at day eight of the hair cycle.

Figure 11: A graph demonstrating the trypsin inhibitory activity of soymilk.

Figure 12: Western blot of C57Bl/6 mouse skins throughout the hair cycle, demonstrating reduced tyrosinase and TRP-1 protein levels following soymilk treatment.

Figure 13: Photographs of untreated and soymilk treated sides of human face, treated with soymilk daily for four weeks.

Figure 14: Quantitative analysis of hair follicle dimensions with and without soymilk treatment.

Figure 15: Photographs of human leg hair following five weeks of soymilk treatment on one leg.

Figure 16: Photographs of control, soymilk, and isoflavone-enriched soymilk treated C57Bl/6 mouse hair (high magnification).

Figure 17: Histological sections of control, soymilk, and isoflavone-enriched soymilk treated C57Bl/6 mouse skins at day 15 of the hair cycle, documenting the thickness and color of the hair shafts.

Figure 18: Photograph of C57Bl/6 mouse hair after three weeks of treatment with various soymilk and isoflavones formulations.

Detailed Description of Preferred Embodiments

As used herein, "mammal" shall mean any member "of the higher vertebrate animals comprising the class Mammalia," as defined in Webster's Medical Desk Dictionary 407 (1986), and includes but is not limited to humans. As used herein "(%, w/v)" shall mean grams of a given component per 100 ml of the total composition.

Topically active agents suitable for use in the composition of the present invention include protease inhibitors and natural plant extracts having protease inhibitory activity and mixtures thereof. Preferred protease inhibitors are serine protease inhibitors, and

in particular Soybean Trypsin Inhibitor ("STI") and the soybean-derived Bowman Birk Inhibitor ("BBI). Preferred botanical extracts are of the legume family and in particular bean extracts, such as soymilk. Preferably, the protease inhibitors are present in an amount, based upon the total volume of the composition of the present invention, of from about 0.0001% (w/v) to about 20% (w/v), and more preferably from about 0.001% (w/v) to about 5% (w/v). Preferably, botanical aqueous extracts such as soymilk are present in an amount of 10-99% (v/v), and more preferably from 50-99% (v/v).

We have unexpectedly found that when topically active agents such as described above, and in particular soymilk or soymilk containing formulations, are enriched with isoflavones, and in particular soybean-derived isoflavones, the inhibitory effect on hair growth, hair dimensions and hair pigmentation is enhanced. Preferably, the isoflavones are present in the botanical aqueous extracts such as soymilk in an amount of 0.000005-15% (v/v), and more preferably from 0.00001-10% (v/v).

If the delivery parameters of the topically active pharmaceutical or cosmetic agent so require, the topically active composition of the present invention may be further comprised of a pharmaceutically or cosmetically acceptable vehicle capable of functioning

as a delivery system to enable the penetration of the topically active agent into the hair follicle and the skin.

The pharmaceutical or cosmetic composition may be optionally combined with other ingredients such as moisturizers, cosmetic adjuvants, anti-oxidants, depigmenting agents, anti-aging agents, hair removal agents, hair styling agents, sunscreens surfactants, foaming agents, conditioners, humectants, fragrances, colorants, viscosifiers, buffering agents, preservatives, and the like and mixtures thereof. These will be combined in an amount which will not affect the serine protease inhibitory activity, in order to produce cosmetic or pharmaceutical products such as, non-exclusively, essences, creams, lotions, pastes, gels, powders, patches or injectables and the like for the reduction of hair growth, hair size and hair pigmentation.

The compositions of this invention may be applied prior to, concurrently with or after other active ingredients or compositions to enhance their effect. For example, the compositions of this invention may be applied in conjunction with one or more products whose purpose is to facilitate the removal of hair to to actually remove hair, reduce hair visibility, improve hair style or improve hair management. The compositions

of this invention may be applied topically prior to, during or following hair removal. They may be applied topically concurrently with one or more of the following group: depilatory agents, shampoo, hair conditioner, styling gel, hair care products, waxing products, shaving products, hair-removal products, after-shave products, deodorant, anti-perspirant, electrolysis, laser hair removal, light-induced hair removal, mask or bath additives.

The compositions of this invention may be applied daily for at least four to eight weeks, by which an effect upon the appearance of hair should be observed. Application may be continued as long as desired to maintain the condition of the hair. Daily application to the face may mitigate the condition of pseudofolliculitis barbae and/or hirsutism; application to the axillary area may reduce hair growth under the arms and application to the scalp and hair may assist in managing and styling African-type hair.

The topically active pharmaceutical or cosmetic composition should be applied in an amount effective to effect changes in mammalian hair growth, hair follicle and hair shaft size and hair shaft pigmentation. As used herein "amount effective" shall mean an amount sufficient to cover the region of skin surface where a delay in hair growth and hair pigmentation and reduced

hair size are desired. Preferably, the composition is applied to the skin surface such that, based upon a square cm of skin surface, from about 2 µl /cm^2 to about 500 µl /cm^2 of topically active agent is present when a delay in hair growth, hair size and hair pigmentation is desired.

We have unexpectedly found that when topically active agents, such as soymilk, or isoflavone-enriched soymilk are topically applied to an animal's skin, a significant delay in hair growth, hair follicle and hair shaft size and hair shaft pigmentation was achieved. We further believe that since the hair growth cycle for humans is often slower than that for mice, it is further likely that the hair growth delay in humans would be considerably longer than in mice.

The invention illustratively disclosed herein suitably might be practiced in the absence of any component, ingredient, or step which is not specifically disclosed herein. Several examples are set forth below to further illustrate the nature of the invention and the manner of carrying it out. However, the invention should not be considered as being limited to the details thereof.

Examples

EXAMPLE 1: *Depilation of Test Subjects in the Mouse System*

C57BI/6 or C3H mice (male and female) were obtained from Charles River (Kingston, NY), at 8 -10 weeks of age and were in the resting (telogen) phase of their respective hair cycle. Hair growth was induced by wax depilation (plucking) of each respective animal's back fur according to the procedure set forth in Stenn, et al., "Glucocorticoid Effect on Hair Growth Initiation: A Reconsideration," 6 Skin Pharmacol. , 125 -134 (1993). In C57Bl/6 and C3H mice, 8-10 weeks old, the growth phase (anagen) starts synchronously in all hair follicles at the time of depilation. As illustrated in Table 1, the following observations were noticed at the induction site:

Table 1: Observations at Induction Site

Days Post-Induction	**Morphological and Histological Observations at the induction site**
1 - 2 (early anagen)	new follicle starts to grow
3 to 4	hair follicles were fully developed, but the hair shafts were not yet visible
7 to 8 (late anagen)	each mouse had very dark skin; their hair shafts are histologically visible
11 - 12	the hair shafts started to penetrate through the epidermis.
14	each mouse was covered with short hairs

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19	the regression of the follicle (catagen) was observed histologically
21 to 25	the hair follicle is back to resting phase.

As shown in Table 1, the hair growth was visible several days after depilation as the pink skin of the animal began to darken. This is likely due to hair pigmentation in the shaft since the C57BI/6 and C3H mice contained melanocytes only in the hair follicles and not in the dorsal epidermis. Similar hair growth pattern was documented in our international application No. PCT/US 97/11033, when chemical depilation using commercially available products was performed.

Since the murine hair cycle varies not only between strains, but also amongst individual animals, the status of the hair cycle was analyzed in each animal on study. A 2 cm by 1 cm skin sample was isolated from each mouse with scissors, fixed with a 10% buffered formalin solution having a pH of about 6.9 - 7.1 at 25 °C (Stephens Scientific), and then formed into a paraffin block according to well-known procedures. The block was then microtomed, and sections were stained with H&E or Fontana-Mason stain. Sections were examined histologically in order to verify the phase of the hair cycle, the size of the hair follicle and hair shaft and the level of hair pigmentation, using procedures well known in the art. Hair length was assessed visually,

and by using a low magnification (x8) dissecting microscope.

This Example, as well as the one described in our international application No. PCT/US 97/11033, shows that the hair growth cycle for C57BI/6 and C3H mice averaged about 25 days and reports similar timing of hair follicle and shaft development regardless of the method used for depilation.

EXAMPLE 2: Preparation of soymilk and soymilk formulations

One way to make soymilk is to soak the soybeans in deionized or purified water for several hours, and grind them after they were fully hydrated, with the addition of small quantities of water. (The grinding process allows the soybean milk to be extracted). After collection, the soybean milk may be filtered to remove any residual parts of the bean husk. The soymilk used in the formulations described below can be fresh soymilk as described above, or may be made from soybean powder and water. The soybean powder is milled from soybeans and may also be lyophilized, spray dried, or freeze-dried and the resulting soymilk may or may not be filtered. Such prepared soymilk may have from about 1 to about 90% by weight dry soybean powder. Another example is the use of soymilk powder, made from lyophilized, spray dried or freeze-dried soymilk, with the addition

of water and finished with or without filtration or homogenization. Other methods of soybean extraction could also be used to create the active ingredients in the formulations described below. For example, the active ingredients could be extracted from ground soybeans using ethanol/water mixtures, followed by the removal of the ethanol from the extract, in such ways that the serine protease inhibitory activity of the soybean will be retained, and preferably that the protein STI will remain intact.

The soy products useful in this invention may be produced from all soybean species, regardless of their geographic origin, sun exposure, harvest time and the like. However, specific strains, geographic origins or growth conditions might be preferred. For example, but not limiting to, soybean strains particularly rich in its Soybean Trypsin Inhibitor (STI) content or in isoflavone content, or growth conditions that result in STI or isoflavone enrichment in the bean, might be preferred. It should be noted that the soy products useful in the compositions of this invention have a distinctive odor, which may be tolerable in some cultures, but is undesired in others. If necessary, the odor of the compositions of this invention may be reduced by using soybean products derived from specific strains of soybeans known to produce reduced-odor, including, but not limited to, lipoxygenase-2-deficient beans and those having modified

sugar profile, and the like. A process to reduce oxygen levels in the formulation may also reduce the odor. Various masking agents or fragrances may also be used to mask the odor.

The compositions of this invention may further comprise surfactants, moisturizers, humectants, conditioners, fragrances, colorants, preservatives, anti-oxidants, depigmenting agents, hair removal agents, anti-aging agents, sunscreens, foaming agents, cosmetic adjuvants, buffering agents or mixtures thereof.

The compositions of this invention may be left on the skin for a period sufficient to effect changes. For example, the compositions of this invention may be applied to the skin daily treatment for at least about four weeks, more preferably, the composition should applied daily for at least eight weeks.

Another method according to this invention is a method to reduce or prevent pseudofolliculitis barbae. Daily application of the compositions of this i nvention may reduce or prevent this condition. The compositions of this invention may also be applied daily to the axilliary area to reduce hair growth. Furthermore, the compositions of this invention may be applied daily to the scalp to style and improve management of African type hair.

As shown in our co-pending U.S. Patent Application ~~(Attorney Docket No. JBP 430)~~ Ser. No. 09/110,409, numerous soymilk-based formulations could be used to reduce pigmentation. All these formulations could also be used to reduce hair growth. Some particularly preferred examples of soymilk formulations and soymilk formulations containing isoflavones are shown in table 2 below. An example for an isoflavones preparation that could be used in this invention is Flavosterone SE from Ichimaru, Japan, which contains about 0.1% pure isoflavones. In all these formulations, soymilk could be replaced with the appropriate quantities of soybean powder or soymilk powder and water.

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Table 2: Soymilk Essence formulations:

Soybean Essences									
	1	6	8	21	22	23	24	25	26
Soymilk	87.42%	89.04%	96.09%	96.05%	96.05%	95.70%	94.40%	94.40%	92.40%
Phenoxyethanol	0.73%								
Phenoxyethanol and Parabens		1.00%	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%
Glycerin	2.50%	2.50%							
Cyclomethicone	2.00%								
Aluminum Starch Ocetyl Succinate	0.75%								
Sucrose Cocoate	1.00%	1.00%							
PEG-6 Capric/Caprylic Triglycerides	3.00%	3.00%							
Disodium EDTA	0.10%	0.10%				0.05%	0.05%	0.05%	0.05%
Polyacrylamide/Laureth-7/C_{13-14} Isoparrafin	2.50%	2.75%	2.90%	2.90%	2.90%	3.20%	3.50%	3.50%	3.50%
Ascorbic Acid		0.01%							1.00%
Butylated Hydroxytoluene		0.10%	0.01%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Polysorbate 20		0.50%							
Lactoferrin							1.00%	1.00%	1.00%
Tocopherol									1.00%
TOTAL	100.00%	100.00%	100%	100%	100%	100%	100%	100%	100%

	27	28	29	30	31	32	33	34	35
Soymilk	90.70%	94.70%	85.70%	90.70%	93.70%	94.70%			
Phenoxyethanol and Parabens	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%
Glycerin	5.00%								
Disodium EDTA	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Polyacrylamide/Laureth-7/C_{13-14} Isoparrafin	3.20%	3.20%	3.20%	3.20%	3.20%	3.20%	3.20%	3.20%	3.20%
Ascorbic Acid									
Butylated Hydroxytoluene	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Deionized Water							90.70%	90.70%	85.70%
Dow Corning 200 Fluid		1.00%							
Flavosterone SE			10.00%	5.00%	2.00%	1.00%			
Soymilk Powder							5.00%		
Soybean Extract using Ethanol/Water Mixture								5%	10%
TOTAL	100%	100%	100%	100%	100%	100%	100%	100%	100%

EXAMPLE 3: ***Preparation of Topically Active Compositions containing soybean derived protease inhibitors***

Soybean trypsin inhibitor (STI) and Bowman-Birk inhibitor (BBI), from Sigma-Aldrich Corporation were mixed into a 0.1M phosphate buffered saline (PBS, Gibco-BRL, Gaithersburg, MA), pH 7.4, in concentrations of 1% to 0.001% (w/v). Four volumes of the resulting solutions were then mixed with 1 volume of (100 mg/ml) liposomes vehicle, which was prepared by the methods described in Niemiec et. al, in order to yield the

topically active composition. Non-ionic liposomes preparations, such as those disclosed in Niemiec *et al.*, "Influence of Nonionic Liposomal Composition On Topical Delivery of Peptide Drugs Into Pilosebaceous Units: An In Vivo Study Using the Hamster Ear Model," 12 Pharm. Res. 1184-88 (1995) ("Niemiec"), which is incorporated by reference herein in its entirety, are well known in the art, and are described our U.S. Patent Application ~~(Attorney Docket No. JBP 430)~~ Ser. No. 09/110,409. GDL liposomes were prepared as set forth in Niemiec, et al., above, with the exception of the following changes: the non-ionic liposomal formulation contained glycerol dilaurate (Emulsynt GDL, ISP Van Dyk)/cholesterol (Croda)/polyoxyethylene-10-stearyl ether (Brij76, ICI)/polyoxyethylene-9-lauryl ether, as at ratio of 37.5:12.5:33.3:16.7. Either PBS or Hepes buffer, 0.05M, pH 7.4 (Gibco-BRL of Gaithersburg, MD) were used as the aqueous phase in the preparation of the liposomes.

EXAMPLE 4: *Soymilk Delays Hair Growth and reduce hair follicle and hair shaft size and Hair shaft Pigmentation*

C57Bl/6 mice were induced for a new hair cycle as described in Example 1, and treated daily with soymilk. Animals were observed daily for their hair growth pattern, and skin biopsies were taken at important time points of the hair cycle. As a result of soymilk treatment the hair growth of the treated mice was delayed, and their hairs were visibly thinner, and

smoother to touch. Treated mice did not show skin darkness at days 7-8 of the hair cycle, as expected, and hair shafts were not visible at days 11-12 as in the control animals. In average, the hair cycle of the soymilk treated mice was delayed by 3-6 days. Figure 1 is a picture of the mice fur, showing the difference in hair appearance, color, size and thickness following soymilk treatment.

Histological examination of the biopsied skin samples confirmed these observations. As shown in Figure 2 by Fontana-Mason (F&M) staining, at day four of the hair cycle the untreated hair follicle is fully developed, as expected, containing all the cellular layers and pigment deposition. In contrast, the soymilk treated sample, (shown at same magnification), shows a smaller and not as fully developed hair follicle, with no pigment deposition.

Figure 3 shows two sets of histological sections stained with F&M, of lower and higher magnification. These sections are from day seven of the hair cycle. The upper panel shows that soymilk treated skin has smaller, shorter, and less pigmented hair follicles than the untreated control. The lower panel shows a higher magnification of the follicles, further demonstrating the difference in hair follicle and hair shaft size and pigmentation following soymilk treatment.

Figure 4 shows low magnification of F&M stained skin sections at day 18 of the hair cycle. At this magnification it is obvious that soymilk treatment results in reduced hair follicle size, which leads to reduced hair shaft length and thickness, and reduced total pigment deposition within the treated follicles.

Figure 5 shows skin sections at day 21 of the hair cycle, with two magnifications. The upper panel demonstrates that the control animals were in the catagen stage, when hair follicles are regressing. Soymilk treated follicles, on the other hand, had already completed the catagen stage, as they are shown in telogen, the resting stage. This indicates that not only the hair cycle was delayed following soymilk treatment, it was also prematurely terminated. The lower panel demonstrates the catagen control follicle and the shorter, telogen (resting) soymilk-treated follicle using higher magnification.

EXAMPLE 5: ***The effects of soymilk on hair growth, size and pigmentation are reproducible in C3H mice***

In order to verify that the effect of soymilk on hair growth is not specific to C57Bl/6 mice, we repeated the experiment described in Example 4 using the brown haired (Agouti) C3H mice. The results of these experiments were similar both visually and

histologically. Soymilk delayed hair growth and reduced hair follicle and hair shaft size and pigment deposition in the C3H mice.

Histological analysis confirmed these visual observations. As shown in the upper panel of Figure 6, using F&M staining, at day seven of the hair cycle soymilk treated follicles are smaller and accumulate less pigment than untreated controls. The upper panel of Figure 7 (F&M staining) shows a lower magnification of the same skin sections, demonstrating the thinner and less pigmented follicles following soymilk treatment.

Figure 8 shows F&M stained skin sections at day 21 of the hair cycle. As shown for the C57Bl/6 mice, following soymilk treatment the hair cycle terminates prematurely. Soymilk treated follicles are in the resting state, while untreated control follicles are still in catagen.

EXAMPLE 6: *The effects of soymilk and soybean derived serine protease inhibitors on hair growth, size and pigmentation*

In search for a mechanism to explain the effect of soymilk on hair growth, we tested the effect of the soymilk-derived serine protease inhibitors, STI and BBI. We had shown earlier that these proteins induce

depigmentation in skin, by affecting the PAR-2 pathway (U.S. Patent Application, ~~Attorney Docket No. JBP 430~~ Ser. No. 09/110,409).

The experiments described in Example 4 were repeated using STI, BBI, and soymilk. STI and BBI were used in a PBS-liposome vehicle as described in Example 3. Visual observations throughout the hair cycle confirmed that both STI and BBI could delay hair growth and reduce hair follicle and hair shaft size, similar to soymilk (see hair pictures in Figure 9). Using high concentrations of STI or BBI, the effect on hair growth and pigmentation was substantial.

Histological analysis confirmed these finding. As shown in Fig. 6, at day seven of the hair cycle 1% of STI and 1% of BBI reduce hair follicle and hair shaft size and hair shaft pigmentation in C3H mice. Figure 7 shows lower magnification sections of the same day into the hair cycle, demonstrating smaller hair follicles and hair shafts and reduced pigmentation, relative to untreated control, with soymilk, STI or BBI treatment. Figure 10 shows that STI and BBI have the same effect in C57Bl/6 mice too, demonstrating smaller and less pigmented follicles. Taken together, this example shows that STI and BBI are soybean-derived serine protease inhibitors, found in soymilk, that could delay hair growth, reduce hair follicle and hair shaft size and reduce hair pigmentation. STI and BBI could represent a

part of the soymilk ingredients that affects hair growth.

In order to support the hypothesis that STI and BBI in soymilk are involved in the hair growth effects described above, we tested soymilk for its serine protease inhibitory activity. An enzymatic assay was performed using "Enzchek", a protease digestion fluorescent test system made by Molecular Probes of Eugene, OR. Using 100 units of trypsin (from Sigma chemicals, St. Louis MO) the test system produced fluorescence reading of about 1100 units. This reaction was inhibited with increasing concentrations of STI, as expected from a known trypsin inhibitor. Serial dilutions of soymilk were tested in this assay, and found to inhibit trypsin activity. As shown in Figure 11, soymilk exerts trypsin inhibitory activity similar to about 0.2% of pure STI. This suggests that soymilk could exert its hair growth effect, at least in part, by STI and BBI.

EXAMPLE 7: *Soymilk Induces Changes in Tyrosinase and TRP-1 protein expression*

The histological analyses of soymilk treated skin samples described in the examples above show dramatic reduction in pigment deposition within the hair follicle. To further understand the mechanism of soymilk-induced depigmentation, we studied tyrosinase,

the key enzyme in melanogenesis and Tyrosinase-Related Protein-1 (TRP-1), the enzyme that stabilizes tyrosinase. C57Bl/6 and C3H mice were treated as described above, and samples were collected throughout the study for protein analysis. Protein extraction and Western blot analysis were performed using standard procedures, such as the one described in Current Protocols in Cell Biology, Edited by Juan S. Bonifacino et al. Chapter 6: Electrophoresis and Immunoblotting. which is incorporated herein by reference in its entirety. An example of one such study is shown in Figure 12.

Equal amounts of skin-extracted proteins were probed with the anti-tyrosinase antibody "anti PEP1", and with the anti-TRP-1 antibody "anti PEP7" which are described in Jimenez, M., Kameyama, K., Maloy, WL, Tomita Y., and Hearing, V. Mammalian tyrosinase: biosynthesis, processing and modulation by melanocyte stimulating hormone. Proc. Natl. Acad. Sci. USA (1988), 85:3830-34, and Jimenez, M., K., Maloy, WL, and Hearing, V. Specific identification of an authentic tyrosinase clone. J. Biol. Chem. (1989) 264:3397-3403, which are incorporated herein by reference in their entirety.

As shown in Figure 12, The expression of Tyrosinase and TRP-1 proteins is dramatically affected by soymilk treatment. Tyrosinase and TRP-1 levels are reduced, and

the duration of the expression is shortened. These two factors affect overall hair pigmentation, which is reduced due to the reduced level and shorter duration of melanogenesis.

EXAMPLE 8: *Soymilk reduces human facial hair length and thickness*

An individual male with dark facial hair who shaves daily was treating the right side of his face with soymilk, immediately after shaving, for five weeks. By the third week, and more noticeably by the forth week, the hair of the treated side was visually lighter and felt smoother to touch. Digital pictures at different magnifications were taken throughout the treatment period, using Hi-Scope. These pictures clearly demonstrate the reduced size and thickness of the hair shafts at the treated side. An example of such pictures is shown in Figure 13, demonstrating the difference in hair shaft thickness and density at four weeks of treatment. Since both sides of the face were shaved at the same time, and pictures of both sides were taken at the same time, the difference in length of the facial hair indicates slower growth rate at the treated area.

Figure 14 shows a computerized image analysis of the facial hair length, thickness and total area, following four weeks of soymilk treatment. All images were analyzed with Image Pro Plus 3.0 software (Media

Cybernetics, Silver Spring, MD). Data are presented as average of 180 hair shafts of each side of the face, with standard deviation (SigmaPlot® 5.0, SPSS Science, Chicago, IL). Statistical analysis was performed using SigmaStat® 2.0 (SPSS Science) software, demonstrating a statistical significant difference in all measured parameters, following soymilk treatment.

EXAMPLE 9: *Soymilk reduces human legs hair length and thickness*

Hair was wax-depilated of two symmetrical areas of the medial part of the legs, below the knee, in one individual. One leg was treated daily, for four weeks, with soymilk. Visual observations indicate slower hair growth on the treated site. Hair shafts were reduced in number and were shorter and thinner than those of the untreated site, as shown in Figure 15. These observations further confirm the effect of soymilk on hair growth. Examples 8 and 9 together confirm that the effect of soymilk on human hair growth is not related to the method of hair removal or to the body part being treated.

EXAMPLE 10: *Soymilk formulations enriched with isoflavones are preferred to soymilk formulations in reducing hair growth and pigmentation.*

The experiments described in Example 4 were repeated, using two formulations described in Table 2

above, Soymilk Essence 23 which is a soymilk-based formulation, and soymilk Essence 30 which is identical to Soymilk Essence 23 except the addition of 5% of a 0.1% isoflavones extract. As shown in Figure 16, mice treated with Soymilk essence 23 show reduced hair growth and nicer hair appearance. This effect was more pronounced with the use of soymilk Essence 30, demonstrating that isoflavone-enriched soymilk formulations are superior to soymilk formulations in reducing hair growth. Figure 17 shows histological skin sections of the treated mice, at day 15 of the treatment. The hair shafts documented in these sections clearly demonstrate the reduction in hair shaft dimensions, the reduced level of pigmentation within the hair shaft, and the increased smoothness of the hair shaft following the Soymilk Essence treatments.

<u>**EXAMPLE 11:** ***Soymilk formulations enriched with isoflavones are preferred to isoflavone formulations which are effective in reducing hair growth and pigmentation.***</u>

The experiments described in Example 4 were repeated, using formulations described in Table 2 above, of soymilk essence with or without increasing concentrations of isoflavones. These Soy Essence formulations were compare to similar formulations, where the soymilk component only was replaced with water. These three sets of formulations (Soy Essence,

isoflavones, Soy Essence containing additional isoflavones) were prepared to test the possibility that isoflavones might be sufficient for the effect observed on hair growth. Figure 18 shows the C57Bl/6 mouse hair following three weeks of topical treatment, as described in example 4. Both untreated control mice and placebo treated mice have long and less "ordered" hair. Soymilk Essence 23 reduces hair growth and leads to a nicer appearance, as described earlier in this application. Soymilk Essence formulations containing 1, 5 and 10% of a 0.1% isoflavones containing extract result in a superior effect on hair growth. However, formulations containing isoflavones but no soymilk demonstrate milder, and not as superior effect as when combined with soymilk. This example demonstrates that soymilk formulations containing isoflavone could reduce hair growth. This example further demonstrates that soymilk formulations containing isoflavones reduce hair growth to a higher degree than formulations containing isoflavones alone.

Example 12: Soy Essence formulations affect human hair growth

The efficacy and irritancy potential of Soymilk Essence 23 and 30 compared to a placebo formulation were examined in a blinded placebo-controlled four-week test with twelve pre-menopausal female panelists ages 29 to 45 by evaluations by the study investigator, self-

assessment by panelists and Hi-scope image analysis. Panelists signed an Informed Consent and were instructed about study procedures and expectations and were asked to shave that night. At the baseline visit the following day, two test lotions were distributed to each panelist (Day 1), a placebo lotion and either soymilk Essence 23 or 30. The lotions were randomly assigned to either the right leg or left leg. The test lotions and placebo were used on the respective legs for the duration of the study with no other lotions used on the lower legs. Panelists were instructed to apply the test lotions twice daily, morning and evenings and were also instructed to try to refrain from shaving their lower legs until after each weekly evaluation. On evaluation days, the investigator visually inspected the panelists' legs for any clinical signs of irritation and compared legs for hair growth attenuation. Self-assessment questionnaires were completed by panelists at each evaluation time point (Weeks 1, 2, 3 and 4). In addition, Hi-scope images (2.5 cm in diameter for each image, KH-2400R, Hirox) were obtained at each time point using a MX-MACROZ lens (Hirox).

No panelists dropped from the study for any product-related reason. No signs of irritation were seen in any of the study panelists at any time point, nor was any irritation reported when self-assessed by panelists at any time point during the study.

For the purpose of hair counts all hair, including "stubble", were counted in the given 2.5 cm field for each panelist at each time point. Results showed a decrease in lower leg hair counts by week 5 for Soymilk Essence 30 and by week 4 for Soymilk Essence 23. The placebo treated legs did not show a change in mean leg hair counts throughout the study although the standard deviations were large. The growth rate was calculated by dividing the length of time (in days) since the panelist last shaved, by the average length of leg hairs for that panelist, which was calculated from the hi-scope images using Image Pro Plus analysis for each panelist at each test site. The results are documented in Table 3, demonstrating that both Soymilk Essences 23 and 30 treatments resulted in reduced hair growth rate compared to placebo.

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Table 3: Mean leg hair growth rates following Soymilk Essence or placebo treatment

Week	Location	Soymilk Essence 30	Soymilk Essence 23	Placebo
0 (baseline)	Upper	0.211 (±0.07)	0.184 (±0.07)	0.243 (±0.07)
	Lower	0.248 (±0.08)	0.191 (±0.06)	0.235 (±0.09)
1	Upper	0.216 (±0.05)	0.153 (±0.03)	0.211 (±0.06)
	Lower	0.178 (±0.08)	0.213 (±0.03)	0.188 (± 0.07)
2	Upper	0.232 (±0.11)	0.181 (±0.04)	0.221 (±0.07)
	Lower	0.236 (±0.12)	0.195 (±0.07)	0.217 (±0.09)
3	Upper	0.241 (±0.06)	0.185 (±0.09)	0.285 (±0.13)
	Lower	0.213 (±0.10)	0.147 (±0.04)	0.253 (±0.12)
4	Upper	0.234 (±0.07)	0.209 (±0.11)	0.211 (±0.09)
	Lower	0.194 (±0.05)	0.208 (±0.07)	0.220 (±0.04)

Results from panelists' self-assessment questionnaires showed that panelists felt that the test lotions attenuated hair growth and softened the feel of leg hair, compared to the placebo lotion. Panelists felt that the hair felt less coarse and less stubbley. The majority of the panelists believed that the test lotions were attenuating leg hair growth or altering the texture of the hair so that it felt smoother and less coarse. Hi Scope analysis further demonstrated that the hair re-growth following treatment with Soymilk Essence 23 or 30 seemed to be growing in the same direction and was more uniform in shape, texture and appearance. In contrast, the hair that re-grew on the

placebo- treated legs grew in different directions, differing in length, angle of growth and thickness.

This Example clearly demonstrate the effect of soymilk formulations in delaying and reducing hair growth, and enabling the growth of softer, less coarse and more managed and directionally-organized hair.